LETTER TO JMG

Q829X, a novel mutation in the gene encoding otoferlin (*OTOF*), is frequently found in Spanish patients with prelingual non-syndromic hearing loss

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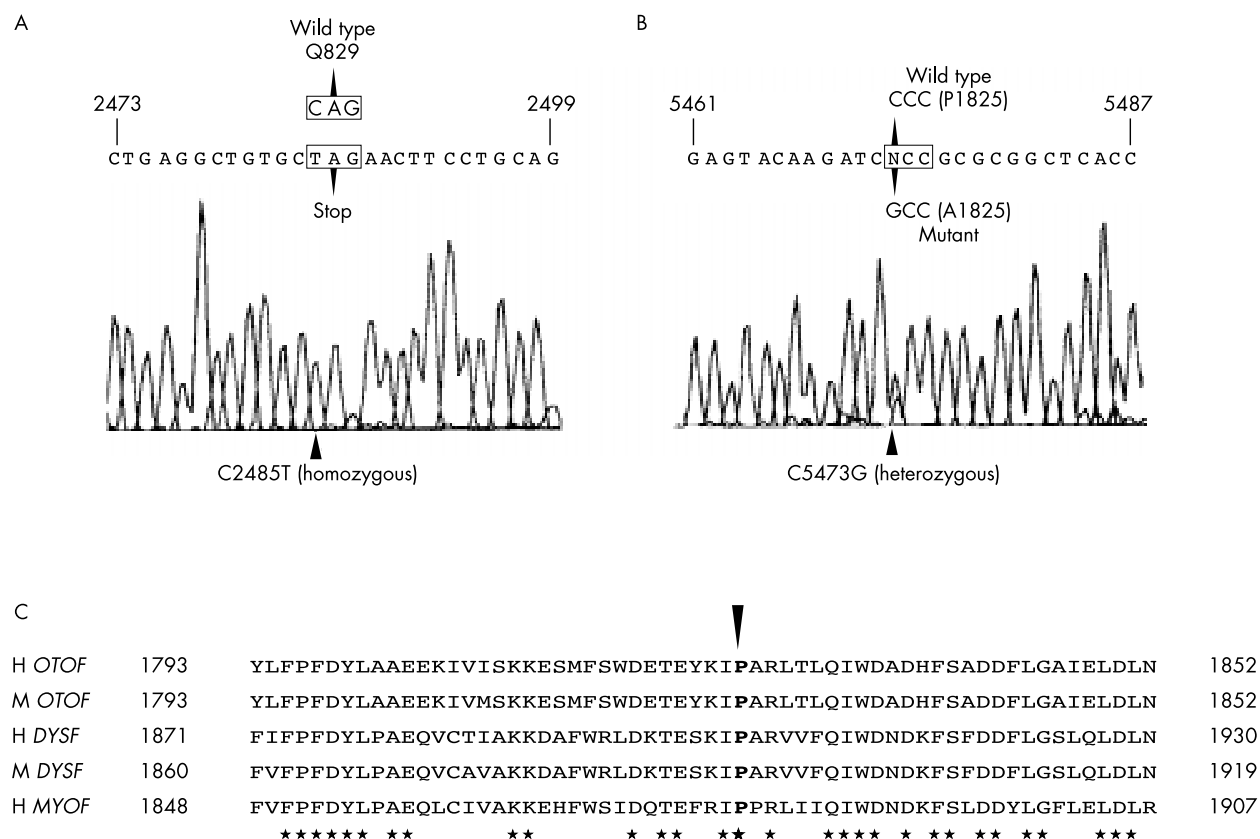
Inherited hearing impairment is a highly heterogeneous group of disorders with an overall incidence of about 1 in 2000 newborns.[1] Among them, prelingual, severe hearing loss with no other associated clinical feature (non-syndromic) is by far the most frequent.[1] It represents a serious handicap for speech acquisition, and therefore early detection is essen-

tial for the application of palliative treatment and special education. Hence genetic diagnosis and counselling are being increasingly demanded.

Non-syndromic prelingual deafness is mainly inherited as an autosomal recessive trait. To date, 28 different loci for autosomal recessive non-syndromic hearing loss have been

Table 1 Sequence of primers used for PCR amplification of human *OTOF* exons

Exon	Forward primer (5′→3′)	Reverse primer (5′→3′)	Size (bp)
1	GCAGAGAAGAGAGAGGCGTGTGA	AGCTGGCGTCCCTCTGAGACA	203
2	CTGTTAGGACGACTCCCAGGATGA	CCAGTGTGTGCCCGCAAGA	239
3	CCCCACGGCTCCTACCTGTTAT	GTTGGGAGTGTAGGTCCCCTTTTTA	256
4	GAGTCCTCCCCAAGCAGTCACAG	ATTCCCCAGACCACCCCATGT	290
5	ACATATACGGGGTGGAGATTGCTGT	CCCGTGTCATGGGAGCAGTG	293
6	ACGTGTCCCCTTGTCTCCTCATTT	CCATGACCCGTGCCAGCTCTA	227
7	GTCCCTCTCCCTGCACCTCATT	TCCTAGAGGGCCACGCATCACT	263
8	CAGCCCACCTAACCAGTCTTTCA	CCGTCCATGAGCCCTGATTCT	267
9	CCTCCTCCCCCACAAGCAGTC	CTAGGAATGCCTCCCAGGAACAAG	281
10	TAGGCTCAGGCAGCTTTCCTTCT	GCCACCCTCCTGCCATATTTACA	231
11	TGGGGCTCAGGGCAAATGTCT	CACGGCGCTGCCTCTTTATCAT	295
12	GCCCTCCCACTTCACCACAAA	AGCCTGCACACCTCGACTGACT	226
13	CGCTCTGGGTGGGGGTGTCT	ACGGGTGGCAGGTGCTCTCA	306
14	TGGCCCTGGCTGTATGTGCT	CCAGGCCCCACACCCATCA	290
15	CATCCCACGCCCTCACCTGT	GGCATCTCACATATTCCTTCCCTTCA	295
16	CGCTGCCCCCTCATTTTTGTT	GCCCTGGGGGACAGCACAGT	292
17	CCGACCTGCCACCCTTACTCAA	GGGTCAACGTTCCCTACAAGAGTGA	299
18	GCCTGGTGGGGAATGCACTCTA	GAGGCGGGAGGTGAGGTCTTG	305
19	CAGCCTCCCATCCTCCTGT	CTGGGCAGACCAGCTTTGT	297
5′UTR	GCTCACCAGAGCCACAGACTCACA	CCTCCCCAGCCTCCCCAAAT	226
20	TGATCAACAGGGAGGAGGCATTT	GCAGGGTCCCCTTTGTCCAGTT	244
23	GGGGCTCAATCAGGAGGCTAGA	CTGCCCCCTCCAGCACCTTA	231
25	GGCTGCCCATCCTGGAACCT	TCACAGGCTTCTGGTGCTCTCAAA	221
26	TGCCCCCCCCCTCTGTCACTT	GCAGGTGGAGTGCAGGGAACAA	256
27	CCAAGAGGCTTCTGGGTTGTCTGTT	CCCAGCCCTAGGCCCCAACT	262
28	GGCAAACAACTCATGGGGAAAGA	TGGTGGGAGGGGGATGACAA	281
29	CATGGCTGGCTCCCTTGACTAA	CTCCAAGAAGGGGCAGAGGAA	307
30	CGGCTTCCTCTGCCCCTTCT	GCTTGGTGGCAGGGTGGATGT	245
31	GCCCCGCCAATCTCACTGTT	GATGTGTCACACGAAGTTGCATGTT	259
32	CCAGGCCCCAGTGGCTGATT	GAGTCCTGCACTCACCCAGCTCTT	250
33	GGTGGAGGCTCTCGGGATTGT	GAAGCTGGACAGGAGGGTCTGAGT	263
34	CTTAAGCAGAGCACATGGTGGACTT	CGGGGAGGAGGAGGCAGAGT	256
35	GGCCTTCCTGCTCTGCTCATTT	CGCCCCCACACACCCTAGAA	232
36	GCAGGTGGGGCAGGACAAA	GGGCAGTGGTGGGAGGTGAGT	287
37	CTACACCCTGACCCACATTTGTCTT	CCAGATGGAGGATGAGGAGACTT	222
38	CTGGCAGGGGCACTGAAGATGA	GCAGGGGAGGGCACCAAGAA	222
39	CTCCCACCCTAGCCAATCCTTAA	TGGAGGCAAAGCAGGCACACT	273
40	CCAGCCCCTCCAAGCCTGT	GCCCCTCCTGGCCTCTGAA	260
41	AGTCCCTAGTCCCAGCAAAGGTCTT	CCCAATACCCAAGAACCCCAGTC	257
42	GGTTAGGAGGGAGAGGAGAGCTGAT	GTTGGGCCGTGGTGGGGAAGT	237
43	CCAAGACCCCAGGGCTTCTCT	CTCCCACCCACAGCCACCTT	300
44	GGCTGCGGGTCTGGAGATGT	CCCATCCCTGCCCTGCTCT	296
45	CACCCCAGCCCTGTTCACTCT	CCAGTCCCAGCCCTGCCTAC	269
46	GGCAGTAGGCAGGGCTGGGACT	GGGTGTCTGGGGATCGTCTCCTT	266
47	CCTCTGCGCTCTCTACCCTTCATA	CCCTTGGTCCAGAGGAAGAAGTAAG	264
48	TGACCGGCTCCTCTCTTCCACTT	GGGAAAGAGTCCAAGCCACTGAAA	279

Figure 1 Novel deafness causing mutations in the *OTOF* gene. (A) Direct sequencing of the coding strand of exon 22 from patient S185-03, homozygous for the Q829X mutation. Nucleotide and amino acid numbers all refer to the long isoforms (accession number AF183185). (B) Direct sequencing of the coding strand of exon 44 from patient S244-05, heterozygous for the P1825A mutation. (C) Comparison of sequences of the sixth Ca2+ binding C2 domain in different members of the mammalian family of proteins sharing homology with *Caenorhabditis elegans* fer-1. H, human; M, mouse; *OTOF*, otoferlin; *DYSF*, dysferlin; *MYOF*, myoferlin. An asterisk denotes residues that are identical in all of the five compared sequences. The proline 1825 residue is indicated in bold. Accession numbers of the compared sequences: AF183185, AF183184, AF075575, AF188290, and AF182316.

reported and 10 genes have been identified.[2] Mutations in the gene encoding connexin-26 (*GJB2*, DFNB1 locus) are responsible for up to 50% of all cases of autosomal recessive deafness, with a frequent mutation (35delG) accounting for up to 86% of the *GJB2* mutant alleles in several populations.[3–10] Other mutations, 235delC and 167delT, account for the majority of *GJB2* mutant alleles among the Japanese[11] and Ashkenazi Jewish populations,[12] respectively. However, little is known about the individual contribution of other genes and their mutations to the remaining uncharacterised cases. Two factors explain this lack of knowledge. First, most of the deafness genes identified so far are large, with many exons and no mutational hotspots, a problem that hampers routine molecular diagnosis. Second, the recent impressive progress in the investigation of genetic deafness has been the result of a research strategy based on the study of large pedigrees with many affected subjects.[13] As a consequence, for most of the genes identified so far, genetic linkage has been reported only for a few families, and a small number of mutations have been published.[14–23] In contrast, most of the families asking for a genetic diagnosis are small, with only one or two affected members, which makes linkage analysis inconclusive. To make the results of the basic investigation available to these small families, research should concentrate on the finding of the most frequent mutations in each separate gene in a given population, and on the development of simple tests, specific for each frequent mutation.

In this study, we have investigated the gene encoding otoferlin (*OTOF*, locus DFNB9 on 2p22-p23)[18] through a strategy based on haplotype analysis of small families followed by

mutation detection in those cases in which the results were compatible with linkage to *DFNB9*. This approach has allowed us to identify a novel mutation, Q829X, the third most frequent mutation causing prelingual deafness reported so far in the Spanish population.

MATERIALS AND METHODS

Twenty-eight independent nuclear families from Spain, with non-syndromic, prelingual, sensorineural hearing loss were enrolled in the study. In six of them, the parents were consanguineous. At least one patient from each of these 28 families had been previously tested for mutations in the connexin-26 gene by heteroduplex analysis and DNA sequencing, the result being negative. Informed consent was obtained from all the subjects included in this study. Clinical examination of the patients excluded syndromic features, as well as putative environmental causes of their hearing loss. Conductive hearing loss was ruled out by otoscopic examination, tympanometry with acoustic reflex testing, and use of the tuning fork tests. Pure tone audiometry was performed to test for air conduction (frequencies of 250-8000 Hz) and bone conduction (frequencies of 250-4000 Hz). DNA was extracted from peripheral blood samples from all members of each family. Subjects were genotyped for microsatellite markers D2S158, D2S2223, D2S2350, and D2S174, close to the *OTOF* gene on 2p22-p23 (genetic distance of 0 cM).[24 25] We used primers and PCR conditions previously reported,[24] but in each case one of the primers was labelled with a fluorescent dye. Amplified alleles were resolved by capillary electrophoresis in an ABI
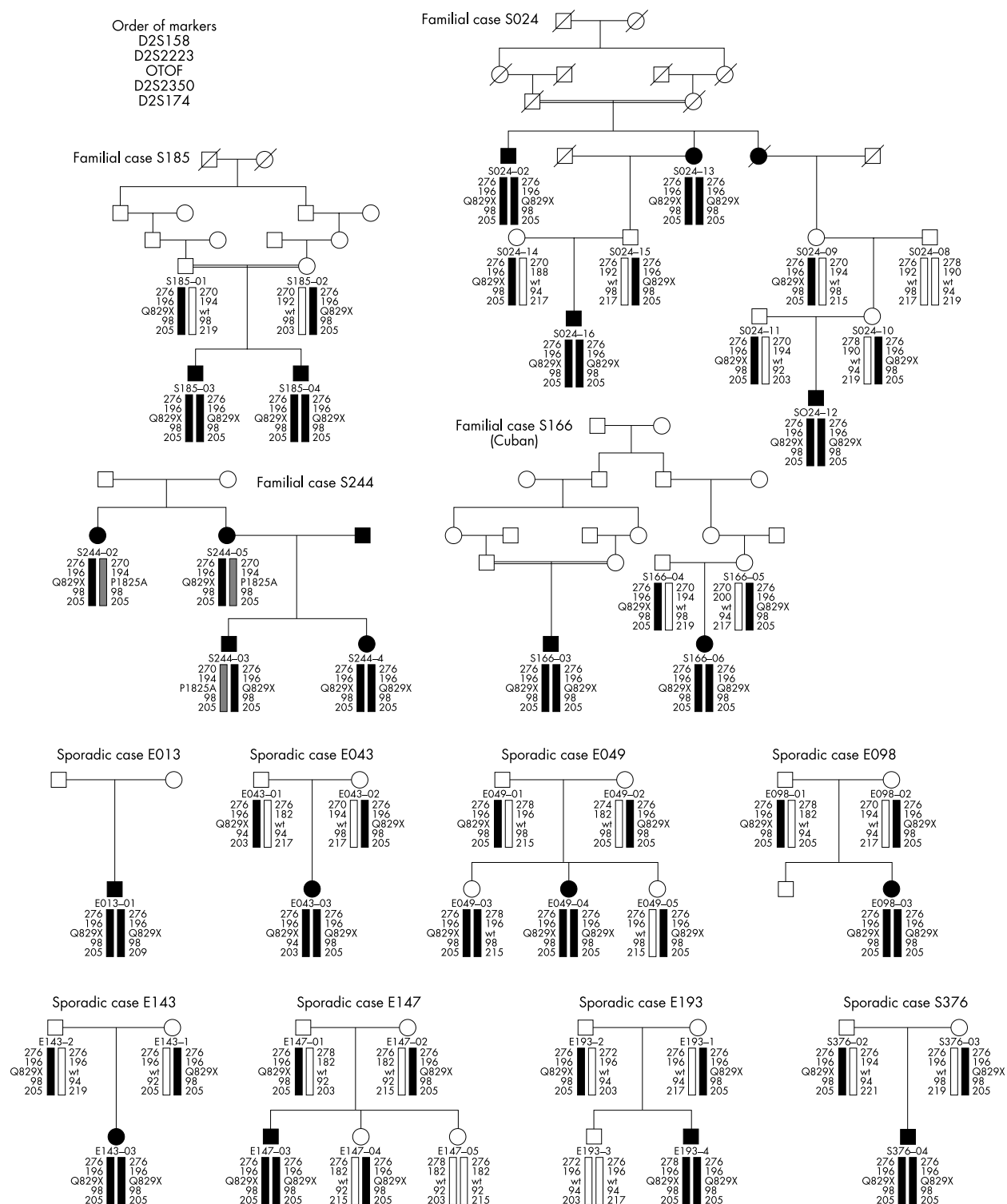
Figure 2 Pedigrees of the familial and sporadic cases carrying mutations in the *OTOF* gene. Haplotypes are indicated by vertical bars. In black, the haplotype associated with the disease. Allele numbers indicate allele size, using as reference CEPH subject 134702.[24]

Prism 310 Genetic Analyzer (Applied Biosystems). The analysis of the haplotype defined for these markers excluded linkage to DFNB9 in 20 families, it was inconclusive in two families, and it showed compatibility with linkage in the remaining six families (including one with consanguineous parents, family S185).

In these six families with putative linkage to DFNB9, an exon by exon screening for mutations in the *OTOF* gene was carried out. Mutation detection was performed by heteroduplex analysis[26][27] of PCR products from one patient and one parent in each family, followed by DNA sequencing of the positive cases. Primers for PCR amplification of each exon have been previously reported.[18][20] However, with the exception of exons 21, 22, and 24, we designed new primers in order to obtain amplification products of the appropriate size for our mutation detection technique (table 1).

RESULTS

In family S185, a C to T transition at nucleotide 2485 was found in exon 22 (fig 1A). This mutation results in the substitution of a glutamine codon by a premature stop codon (Q829X). Both parents of family S185 were carriers of the mutation and their two affected children were homozygous (fig 2). The Q829X mutation was not found in 200 unrelated Spanish controls with normal hearing. No other pathogenic mutation was detected in the remaining five families, but the screening showed several polymorphic changes in the *OTOF* open reading frame: six of them are silent (A372G, G945A, C2580G, G2736C, C5391T, and C5655T) and two others produce amino acid substitutions (C244T and C2317T). The C244T polymorphism results in the substitution of arginine 82 by a cysteine residue (R82C). It was found in seven out of 10 unrelated controls with normal hearing. On the other hand, the C2317T polymorphism results in the substitution of arginine 773 by a cysteine residue (R773C), and it was found in eight out of 46 unrelated controls with normal hearing.

A specific test was designed for easy screening of the novel Q829X mutation. Exon 22 was PCR amplified using previously reported primers. The amplification product (298 bp) was then digested with the *Bfa*I restriction endonuclease, according to the manufacturer's instructions (New England Biolabs), and the digestion fragments were resolved by electrophoresis on a 3% agarose gel (fig 3). In the wild type allele there is one *Bfa*I site, digestion resulting in two fragments of 291 bp and 7 bp. The Q829X mutation creates an additional restriction site, which divides the 291 bp fragment into two parts (168 and 123 bp). We used this test to screen a collection of 269 unrelated cases of autosomal recessive hearing loss negative for mutations in the connexin-26 gene. These included 93 familial cases and 137 sporadic cases from Spain and 16 familial and 23 sporadic cases from Cuba. A case was considered sporadic when there was only one affected person in the pedigree. We found the Q829X mutation in 11 cases: two Spanish familial cases, eight Spanish sporadic cases, and one Cuban familial case. The mutation was found in homozygosity in all of these cases, except in the Spanish family S244 (fig 2), in which there were homozygous and heterozygous patients. So an exon by exon screening for mutations in the *OTOF* gene was performed on one of the heterozygous patients from family S244. A C to G transversion at nucleotide 5473 was found in exon 44 (fig 1B). This mutation results in the substitution of proline 1825 by an alanine residue (P1825A), and it was not found in 100 unrelated Spanish controls with normal hearing. The mutation was also not found in the 258 unrelated cases of recessive deafness that remained uncharacterised after the Q829X screening.

Given the frequency of the Q829X mutation in our sample, we investigated its evolutionary origin. All the families carrying the Q829X mutation were genotyped for microsatellite markers D2S158, D2S2223, D2S2350, and D2S174, and the corresponding haplotypes were deduced (fig 2). Considering the consanguinity observed in three of these families, there are 24 independent chromosomes carrying the mutation (22 Spanish and two Cuban). In 20 Spanish and in the two Cuban chromosomes, the Q829X mutation is associated with the 276-196-98-205 haplotype, which is not represented among the 25 wild type chromosomes from the families included in the study. In two other cases, the mutation is associated with haplotypes 276-196-98-209 and 276-196-94-203 that may be recombinant derivatives of the most common Q829X associated haplotype. These results strongly suggest that the chromosomes carrying the Q829X mutation share a common ancestor. This founder effect would not be the consequence of geographical isolation, since our families with the Q829X mutation are distributed all over Spain and Cuba.
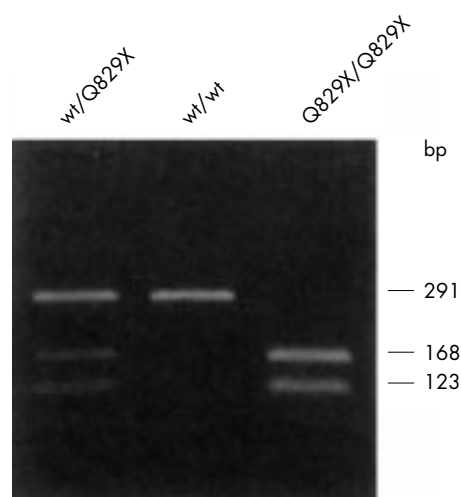

Figure 3 Specific test for the Q829X mutation. *Bfa*I digests of exon 22 PCR products from a heterozygote and both types of homozygotes, resolved on a 3% agarose gel. Fragment sizes in base pairs (bp) are indicated on the right.

DISCUSSION

The *OTOF* gene contains 48 exons and encodes multiple long and short isoforms, owing to alternative splicing combined with the use of several translation initiation sites.[20] The first 19 exons are exclusive of the long isoforms. The encoded protein, otoferlin, belongs to a family of mammalian proteins sharing homology with *Caenorhabditis elegans* fer-1. The members of this family, which also includes dysferlin and myoferlin, are membrane anchored cytosolic proteins that contain six predicted C2 domains, the last four of which are expected to bind Ca2+. The otoferlin long isoforms also have six C2 domains, whereas the short isoforms just contain the last three domains.[18 20] Before this report, only four different mutations in *OTOF* have been reported, all of them homozygous in the patients: Y1497X (formerly Y730X, referring to the short isoforms), found in four unrelated, consanguineous families from northern Lebanon[18]; IVS24+1G>A (reported as IVS5+1G>A, in reference to the short isoforms) found in a consanguineous Druze family[19]; IVS8-2A>G, found in a consanguineous family from India[20]; and R237X, found in a consanguineous family from the United Arab Emirates.[28] The first two mutations affect both the long and the short isoforms, the two others affect only the long isoforms. The mutations reported here, Q829X and P1825A, are the first to be found in a western population and in both consanguineous and non-consanguineous families. They affect both the long and the short isoforms. The P1825A mutation is the first missense mutation found in the *OTOF* gene. It alters a conserved proline residue in the sixth C2 domain of the long isoforms (third in the short ones), a domain that is expected to bind Ca2+ (fig 1C).[20] This mutation appears to be rare in the Spanish population. In contrast, the Q829X mutation is responsible for 12 of 270 cases (4.4%) of recessive (familial or sporadic) prelingual deafness negative for connexin-26 mutations, and about 3% of all cases of recessive prelingual deafness in the Spanish population. This makes Q829X the third most frequent mutation causing recessive deafness in the Spanish population, and suggests a relevant contribution of the *OTOF* gene to prelingual deafness, since other mutations may remain undetected. Consequently, our laboratory has included the detection assay for Q829X in the set of tests routinely performed for the molecular diagnosis of deafness.

Currently, we can provide a molecular genetic diagnosis for about 40% of the Spanish families with recessive deafness who ask for a genetic study. The methodological approach

used in this work has proved to be a useful tool for the discovery of novel, frequent mutations in small families. Its application to other genes for recessive deafness should contribute to finding a selected group of frequent mutations whose routine diagnosis would be quick and easy, allowing us to increase the percentage of diagnosed cases to reach figures comparable to those of other pathologies.

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